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Mycoremediation of acetaminophen: Culture parameter optimization to improve efficacy

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HIGHLIGHTS

- Adjustment of pH slightly improved APAP remediation by *Mucor hiemalis.*
- *Phanerochaete chrysoporium* exhibited better APAP remediation without pH adjustment.
- Co-cultivation was less effective than *P. chrysosporium* in monoculture.

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ABSTRACT

Untreated pharmaceutical pollution and their possibly toxic metabolites, resulting from overloaded wastewater treatment processes, end up in aquatic environments and are hazardous to the ecosystem homeostasis. Biological wastewater remediation could supplement traditional methods and overcome the release of these biologically active compounds in the environment. Mycoremediation is especially promising due to the unspecific nature of fungi to decompose compounds through exoenzymes and the uptake of compounds as nutrients. In the present study, we improved on the previous advances made using the fungus *Mucor hiemalis* to remediate one of the most commonly occurring pharmaceuticals, acetaminophen (APAP), at higher concentrations. The limitation of nitrogen, adjustment of pH, and comparison to, as well as co-cultivation with the white-rot fungus *Phanerochaete chrysosporium,* were tested. Nitrogen limitation did not significantly improve the APAP remediation efficiency of *M. hiemalis*. Maintaining the pH of the media improved the remediation restraint of 24 h previously observed. The APAP remediation efficiency of *P. chrysosporium* was far superior to that of *M. hiemalis,* and co-cultivation of the two resulted in a decreased remediation efficiency compared to *P. chrysosporium* in single.

1. Introduction

Pharmaceuticals have improved the quality of life and increased the longevity of humanity. However, ease of availability, improper disposal, and substantial overuse have resulted in the discharge of many pharmaceutical residues. Consequently, pharmaceutical pollution is a formidable challenge for wastewater treatment plants. This pharma-burden is likely only to increase in the future due to a growing and ageing global population. The removal rates of pharmaceuticals in wastewater treatment plants (WWTP) vary considerably depending on the deployed treatment processes leading to substantial amounts of pharmaceuticals being discarded into the environment (Jones et al., 2007; Vieno, 2007; Mankes and Silver, 2013; Nunes et al., 2014; Ternes, 1998, 2001; PILLS project, 2012; Vieno and Sillanpää, 2014; Tran et al., 2014). The presence of these biologically active substances and even their metabolites in the aquatic ecosystem causes adverse effects on biota such as endocrine disruption, decreased motility, feeding difficulties, and altered sex functions and behaviours (Bedner and MacCrehan, 2006; Petrie et al., 2014; UNESCO and HELCOM, 2017), thereby threatening biodiversity and upsetting the ecosystem balance.

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Therefore, action is urgently required to avoid future species loss. These pharmaceutical pollutants, which bioaccumulate in the environment, negatively affect not only nature but can also impact on humans as consumers and recreational users (Daughton, 2008). The economy could also be affected due to decreased yields for fisheries causing revenue loss.

One of the pharmaceuticals frequently detected in the environment is the common over-the-counter analgesic, acetaminophen (APAP), commonly known as paracetamol or according to IUPAC N-(4-hydroxyphenyl)acetamide (Bessems and Vermeulen, 2001). Up to 5 μg/L APAP has been detected in surface and groundwater in close proximity to sewage catchments in Singapore (Tran et al., 2013), a maximum of 10 μg/L in the USA (Kolpin et al., 2002), 108 μg/L in the WWTP effluents and 8.3 μg/L in drinking water wells in France (Rabiet et al., 2006), and up to 1.5 μg/L in rivers in the South of Wales (Kasprzyk-Hordern et al., 2008). Of the mentioned studies, Rabiet et al. (2006), Kasprzyk-Hordern et al. (2008), and Tran et al. (2013) investigated pharmaceutical residues in surface water near WWTP and explicitly pointed out the insufficient removal ability of conventional WWTP. Thus, the development of alternative, complementary methods for WWTP is urgently needed to address this growing global issue. To date, chemical and physical treatment methods have not been useful in the treatment of pharmaceutical waste, especially in large water bodies (Guo et al., 2017). Additionally, treating anthropogenic chemicals by adding more chemicals could even further enhance the problem. Therefore, a nature-based solution could be vital in addressing this issue.

Fungi as decomposers in aquatic environments play a greatly underestimated role and show significant capabilities to degrade organic material. Mycoremediation exploits this ability to degrade persistent xenobiotics by biodegradation, biosorption, and bioconversion (Kulshreshtha et al., 2014). Due to the unspecific nature of their exoenzymes, fungi could also perform a major role in the biotransformation and biodegradation of organic pollutants in aquatic systems. Macromycetes, aka mushrooms, were previously proven efficient in the remediation of some pharmaceuticals (Marco-Urrea et al., 2010; Migliore et al., 2012; Cruz-Morató et al., 2014; Muszyńska et al., 2019). However, their application in practical terms for industrial and biotechnological uses in an aquatic setting may be challenging. Few studies have explored the use of micromycetes, which could have a broader purpose in biotechnological applications. For instance, filamentous micromycetes can be cultivated as small spherical pellets of intertwined hyphae as shown for *Mucor hiemalis* (Balsano et al., 2016) with multiple advantages for application in bioreactors.

Micromycetes are recognized for their ability to adapt to severe environmental constraints and the production of various metabolites and enzymes which are useful in the bioremediation of organic and inorganic compounds and metals (Gupta et al., 2011; Gonzales-Abradelo et al., 2019; Khan et al., 2019; Ariste et al., 2020). The *Mucor* spp. Have been employed in the remediation of heavy metals (Shroff and Vaidya, 2011; Hoque and Fritscher, 2016; Zhang et al., 2017) and organic compounds such as polycyclic aromatic hydrocarbons (PAH) (Jia et al., 2016) and pentachlorophenol (Szewczyk and Długoński, 2009) in monoculture, as well as the herbicide isoproturon co-cultivated with *Phanerochaete chrysosporium* (Hoque, 2003; Rønhede et al., 2005). *Mucor* spp. Are known to express high levels of extracellular glutathione S-transferases (GSTs), a class of detoxification enzymes, which plays a key role in its degradation abilities (Hoque, 2003; Hoque et al., 2007).

M. hiemalis grows efficiently at room temperature (between 20 °C and 25 °C) in aerobic conditions (Schipper, 1973) and tolerates low temperature (mean temperature 10 °C) (Hoque et al., 2007). In particular, *M. hiemalis* EH5 can reproduce even at the low groundwater temperature of 5 °C. It can tolerate a wide pH range of 3–11, which makes it suitable for the purification of ground- and surface water, wastewater, and waters from mines season-independently and under extreme conditions (Fritscher et al., 2005). The micromycete *M. hiemalis* seems to be xenobiotic resistant and has excellent biosorption abilities (Balsano et al., 2015, 2017; Dan et al., 2006).

M. hiemalis f. Irnsingii strain EH5 (DSM 14200; Zygomycota) was previously used for the remediation of diclofenac (Esterhuizen-Londt et al., 2017), and APAP (Esterhuizen-Londt et al., 2016a, b). Between 90 and 97% of diclofenac (10–50 μg/L) in the fungus's environment was remediated within six days (Esterhuizen-Londt et al., 2017). However, for APAP, after 24 h of exposure to environmentally relevant concentrations (up to 20 μg/L APAP), *M. hiemalis* was able to degrade, internalize, and bioaccumulate only up to 50% of the APAP it was exposed to without the amplified oxidative stress. However, after 24 h, remediation halted, possibly due to the environment becoming too acidic. For higher APAP concentrations (100 μg/L and more), the remediation efficiency dramatically decreased even within the first 24 h. Therefore, *M. hiemalis* can be used for the potential remediation of APAP; however, only once the limiting barrier has been recognized and overcome.

Another option is co-cultivation, as shown by several other studies, which increases the remediation capacity of a micromycete observed in monoculture (Hoque, 2003; Rønhede et al., 2005; Dan et al., 2006). For this purpose, the basidiomycete *P. chrysosporium,* considered as a model mycoremediation microbe, was of interest due to its ability to produce a variety of extracellular enzymes (Martinez et al., 2004; Rabinovich et al., 2004). *P. chrysosporium* has been successfully employed to treat numerous pollutants (Sutherland et al., 1991; Li et al., 2016; Rodarte-Morales et al., 2012). However, the fungus grows optimally at warm temperatures ranging from 25 °C to 30 °C in aerobic conditions (Lamar et al., 1987), limiting its real-life application in WWTP. Therefore, the low-temperature tolerant species *M. hiemalis* could potentially function as a useful complement to *P. chrysosporium*.

An essential factor for the growth and normal metabolism of fungi is nitrogen. This is evidenced by the rapid response to changes in nitrogen availability with alterations in the fungal metabolization process (Tudzynski, 2014). Therefore, the role of nitrogen limitation on mycoremediation needs to be evaluated to understand its impact during implementation in real scenarios and to understand if the fungi could use xenobiotics from its environment when starved for nitrogen.

In the present study, the aim was to investigate if the previous APAP remediation potential of *M. hiemalis* could be improved by evaluating the role of pH, nitrogen limitation, and co-cultivation with the white-rot fungus *P. chrysosporium* (Basidiomycota) in order to improve its remediation properties and further evaluate it as a candidate for use in practice.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich unless stated otherwise. The chemicals for quantitative analysis were analytical grade.

2.2. Fungal cultivation

The fungal species *M. hiemalis* f. Irnsingii, strain EH5 (DSM 14200) and *P. chrysosporium* were obtained from the German Collection of Microorganism and Cell Cultures (DSMZ). Sub-

cultured stocks for each fungus, younger than 3rd generation, were used for the experiments.

Fresh spore stocks were aseptically prepared in distilled water from the individual fungal species cultivated on Sabouraud dextrose (SAB, S3306 Fluka) plates for three weeks (Balsano et al., 2016). The spore stocks were enumerated using a Neubauer hemocytometer and an Olympus CH-2 bright field microscope.

The pelletization of the two fungi was developed in liquid SAB medium. The medium was aseptically prepared according to the manufacturer's instruction. The *M. hiemalis* was aseptically cultured in pellet morphology, according to Balsano et al. (2016), i.e. in SAB (pH 7.5 ± 0.5) with an inoculation density of 10^3 spores/mL agitated continuously at 130 rpm for seven days. *P. chrysosporium* was aseptically pelletized by cultivation in SAB (pH 5.6 ± 0.2) at an inoculum concentration of 10^5 spores/mL on a rotary shaker at an agitation speed of 150 rpm and a temperature of 30 °C for four ± one days.

2.3. Cultivation parameters tested

Culture factors influencing APAP remediation were evaluated, optimizing one parameter at a time and keeping the other factors unaltered. Firstly, *M. hiemalis,* as an axenic unifungal culture, was exposed to a fixed concentration of APAP (250 μg/L APAP) in a nutrient-rich (SAB) medium, as a benchmark for the "current" remediation capacity. It was then exposed to APAP in a nutrient-poor (Nutrient limiting medium, N-Lim; Kirk et al., 1978) medium in parallel, with corresponding controls, in order to investigate the effect of starving. The APAP remediation abilities of *P. chrysosporium* was then compared to that of *M. hiemalis* in a nutrient-poor medium, followed by evaluating the effect of pH manipulation. Finally, the two species were cultivated together (co-culture) to test the effect on APAP remediation in conjunction with pH adjustment.

Up to 108 μg/L has previously been detected in the WWTP effluents (Rabiet et al., 2006); therefore, the exposure concentration of 250 μg/L was selected to assess whether the mycoremediation technology could be implemented in WWTP and whether they would be able to cope with maximum real-life concentrations.

2.3.1. Nitrogen limitation

Twenty pellets of *M. hiemalis* were exposed to 250 μg/L APAP ($n = 4$) in SAB media (120 mL, pH 7.5) in Erlenmeyer flasks. The same experimental set was prepared with N-Lim medium (Kirk et al., 1978), consisting of 0.2 g/L $CaCl_2$, 0.103 g/L $NaHCO_3$, 0.1 g/L sea salt, 5 g/L glucose, 0.05 g/L NH_4Cl, in parallel ($n = 4$). The controls consisted of APAP in SAB or N-Lim in the absence of fungal pellets. The cultures were incubated at 20 °C ± 2 °C with an agitation speed of 130 rpm. Media samples were taken after 24 h, 48 h, 72 h, and 168 h; the pH was recorded, and then the samples were analyzed using liquid chromatography-tandem mass spectroscopy (LC-MSMS).

2.3.2. pH adjustments with M. hiemalis

Because of the significant drop in pH recorded in the previous experiment, pH was adjusted to see if it poses as a limiting factor in the remediation efficiency of *M. hiemalis*. Due to the results obtained with N-Lim and the fact that in surface waters conditions representing a rich medium such as SAB is implausible, the rest of the experiments were conducted only in N-Lim.

M. hiemalis pellets (20 pellets) were incubated in N-Lim with a starting pH of 7.5 ± 0.5 and an APAP concentration of 250 ± 25 μg/L ($n = 4$). In one experimental set, the pH in N-Lim was evaluated without adjustment (i.e. only when the medium was prepared), and in the second set, the pH of the medium was adjusted daily to 7 ± 0.5. Each experimental set-up consists of a treatment (fungi pellets) and a control (no fungal pellets). The samples were taken after 0 h, 24 h, 48 h, 72 h, and 168 h after the inception of the experiment to measure the pH value and analyze the APAP concentration via LC-MSMS. The cultures were incubated at 20 °C ± 2 °C with an agitation speed of 130 rpm.

2.3.3. pH adjustments with P. chrysosporium

The pellets produced using *P. chrysosporium* were much smaller (1–2 mm) than those using *M. hiemalis* (3–4 mm). Therefore, the biomass versus pellet number was assessed to determine the number of *P. chrysosporium* pellets which equals 20 *M. hiemalis* pellets in terms of biomass. The biomass was quantified by weighing the pellets after lyophilization (−50.2 °C, 0.1043 mbar for 24 h in a Lio 5 P, Kambič Laboratorijska oprema). Thus, 20 *M. hiemalis* pellets equalled 59 *P. chrysosporium* pellets ($y = 0.512x - 10.43$, $R^2 = 1$; where y represents *Mucor* pellets, and x represents *Phanerochaete* pellets) in terms of biomass. Therefore, 59 pellets were incubated in 120 mL of N-Lim media (pH 7.5) containing APAP at a concentration of 250 ± 25 μg/L ($n = 4$), in two sets, with corresponding controls, i.e. which lacked fungal pellets, to assess the natural degradation of APAP. For one set, the pH was not adjusted, and for the second set, the pH was adjusted daily to 7.5 ± 0.5 after taking samples for LC-MSMS analysis and pH measurement. As before, samples were taken after 0 h, 24 h, 48 h, 72 h, and 168 h. The cultures were incubated at 30 °C ± 2 °C with an agitation speed of 150 rpm.

2.3.4. Co-cultivation

M. hiemalis and *P. chrysosporium* were individually pelletized as stipulated before. Ten *M. hiemalis* pellets together with 40 *P. chrysosporium* pellets were then aseptically introduced into 120 mL of N-Lim medium containing 250 ± 25 μg/L APAP ($n = 4$) in two experimental sets, the first with no pH intervention and the second pH-adjusted daily to 7.5 ± 0.5 after taking samples for LC-MSMS analysis and pH measurement. As before, samples were taken after 0 h, 24 h, 48 h, 72 h, and 168 h. Flasks were incubated at 28 °C and continuously shaken at 150 rpm for seven days. The chosen incubation temperature for co-cultivation was an average of the optimal temperature needed for the growth of the two types of fungi in single (i.e. *M. hiemalis* at 25 °C and *P. chrysosporium* at 30 °C). The pellet numbers were based on the biomass of 20 *M. hiemalis* pellets shared equally by the two species.

2.4. Quantitative analysis

The media samples, collected at the various time points as described, were analyzed on LC-MSMS, to assess the APAP concentration. Chromatographic separation of APAP was achieved on a Kinetex C18 column (2.6 μm, 2.1 × 100 mm) by liquid chromatography (1200 infinity Series, Agilent, Waldbronn, Germany) with a gradient consisting of 10% acetonitrile in water (solution A) and 0.1% formic acid in water (solution B) at a flow rate of 0.2 mL/min. For the first minute, solution A was set to 10%. It was linearly increased to 100% A within 4 min and kept constant for 1 min. Then, solution A was decreased to 10% within 5 s, followed by post-run for 2 min. The injection volume was 10 μL and the retention time of APAP was 4.43 min. The LC was coupled to triple quadrupole mass spectrometry (model 6460 Triple Q™, Agilent) with electrospray ionization (Jet-Stream, Agilent). The conditions were set as follows: capillary voltage 3.5 kV, gas temperature 300 °C, gas flow 10 L/min, nebulizer 35 psi, sheath gas temperature 350 °C, and sheath gas flow 11 L/min. MS-MS was conducted in the positive MRM mode with a mass transfer of 152 (Q1) and 65 (Q2) and 93 (Q3) for APAP (Esterhuizen-Londt et al., 2016b). The calibration curve was linear

($R^2 = 0.999$) between 0.1 μg/L and 500 μg/L (nine points, six replicates) with a limit of detection (LOD) of 0.08 μg/L and a limit of quantification (LOQ) of 0.25 μg/L.

2.5. Statistical analysis

Statistical analyses were performed using IBM® SPSS® statistics version 25 (2017). A factorial repeated measures (Mixed Between-Within Subjects) analysis of variance (ANOVA) was performed to assess the effects of time, treatment, and the interaction of the two factors. Levene's, Sphericity, and Boxes tests were considered to assess the normality and homogeneity of the data before further assessment. The main effects were compared with the Bonferroni post-hoc test.

3. Results and discussion

3.1. Nitrogen limitation

Theoretically, when limiting the medium nitrogen, it is expected that an organism would be forced to use compounds from its environment as a nitrogen source, such as xenobiotics, which would, in this case, be APAP. Therefore, this was tested by using the N-Lim medium (Kirk et al., 1978), which contained 10-fold less nitrogen compared to SAB.

Nitrogen limitation caused significant APAP removal compared to the control from day one compared to the APAP removal only being significant after the seventh day in nutrient-rich media. Compared to the initial APAP concentration in SAB (Fig. 1A), 15.6% was removed after seven days; however, this was not significantly lower than the degradation seen in the control ($p = 0.098$). Cultivation in N-Lim medium (Fig. 1B), resulted in a similar removal percentage (14.5%); however, remediation in the presence of the fungus was significantly lower compared to the corresponding control in N-Lim after the seventh day ($p = 0.005$). WWTP influent is typically nutrient-rich; however, after the tertiary treatment processes, where bioremediation treatments are likely to be implemented, the waters contain much less nutrients. Based on this, all further experiments were conducted in N-Lim media.

When the nitrogen available to a fungus in its environment is limited, it needs to invest energy into extracellular enzyme production to obtain it. However, the pay-off is lower growth efficiencies (Lonardo et al., 2020). As seen for *Mucor* spp., nitrogen, or the limitation thereof regulates enzyme production (Yazdi et al., 2006). The presence of a nitrogen-containing chemical (xenobiotic) in the environment usually functions as an inducer (Monica et al., 2014); APAP in the present study, leading to a significant decrease compared to the control after the seventh day.

3.2. pH adjustments with M. hiemalis

Evaluating the medium pH over time in the presence and absence of *M. hiemalis* in SAB (Fig. 2A) and N-Lim media respectively (Fig. 2), revealed that there is a significant drop in pH over time compared to the control for both medium types ($p < 0.001$). It can be concluded that the fungus (treatment) strongly affects the pH level and that the natural dissolution of APAP alone (control) does not significantly influence the pH level for the treatment samples. The reduced pH is likely due to the production of organic acids and the removal of ammonium ions from ammonium sulfate salt or excretion of H^+-ions as a byproduct of NH_4^+ assimilation (Prusky et al., 2001; Bi et al., 2016).

Since the APAP remediation efficiency declined after the first 24 h (as previously reported by Esterhuizen et al., 2016a), coinciding with the sharp pH drop observed in SAB after 24 h, pH was evaluated as a limiting factor. Various enzymes function at different pHs, e.g. proteases have an optimal pH of between 3 and 3.5 (Wang et al., 1967).

Compared to the initial APAP concentration, 25.5% ($p < 0.001$) was remediated after seven days (Fig. 3A) with daily pH adjustment back to 7.5 (Fig. 3B). The APAP concentration in the presence of *M. hiemalis* pellets compared to the sterile control medium was significantly lower after 24 h. By maintaining the pH at 7.5, the APAP remediation by *M. hiemalis* was improved by 12% (Fig. 3A) compared to when unadjusted ($p = 0.021$) (Fig. 1B).

The enzyme glutathione *S*-transferase enzyme (GST) produced by *M. hiemalis* is active from pH 6 to 9 and has a pH optimum between 7.5 and 8 (Irzyk and Fruest, 1993). Other enzymes of *M. hiemalis* such as antioxidative enzyme catalase (CAT), and glutathione reductase (GR) are active over the pH ranges of 4.0 and have their optimum activity over the pH of 6.5–7.0 (Halliwell and Foyer, 1978; Aebi, 1984). Therefore, within the daily pH variations

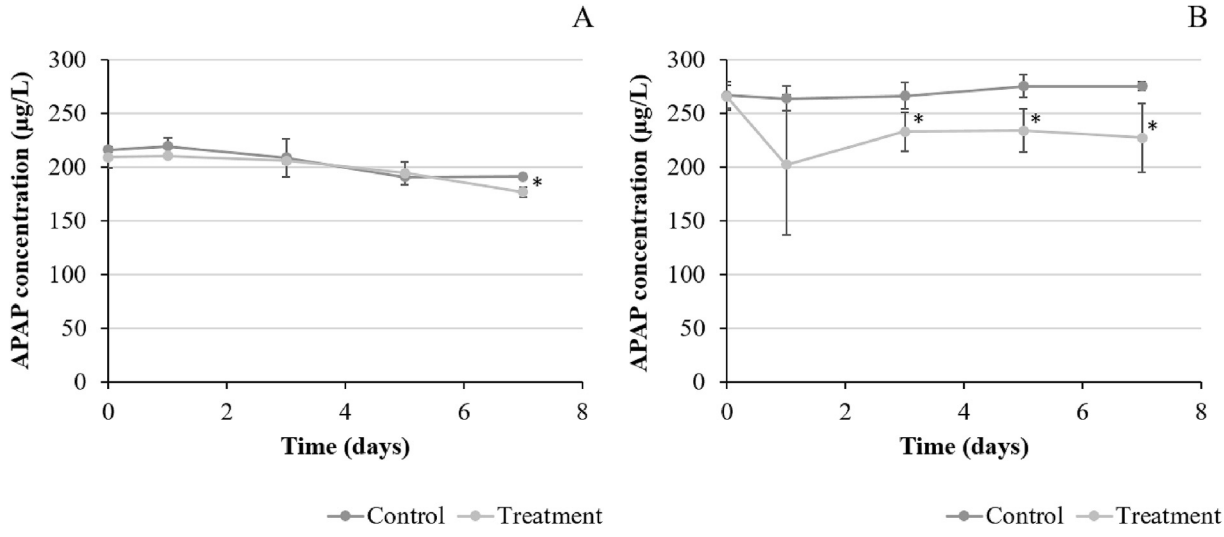

Fig. 1. APAP concentration in A) SAB media and B) N-Lim media in the presence of *M. hiemalis* pellets (treatment) and sterile media (control). Asterisks (*) represent statistical significances compared to the control. Data are presented as mean ± standard deviation ($n = 4$).

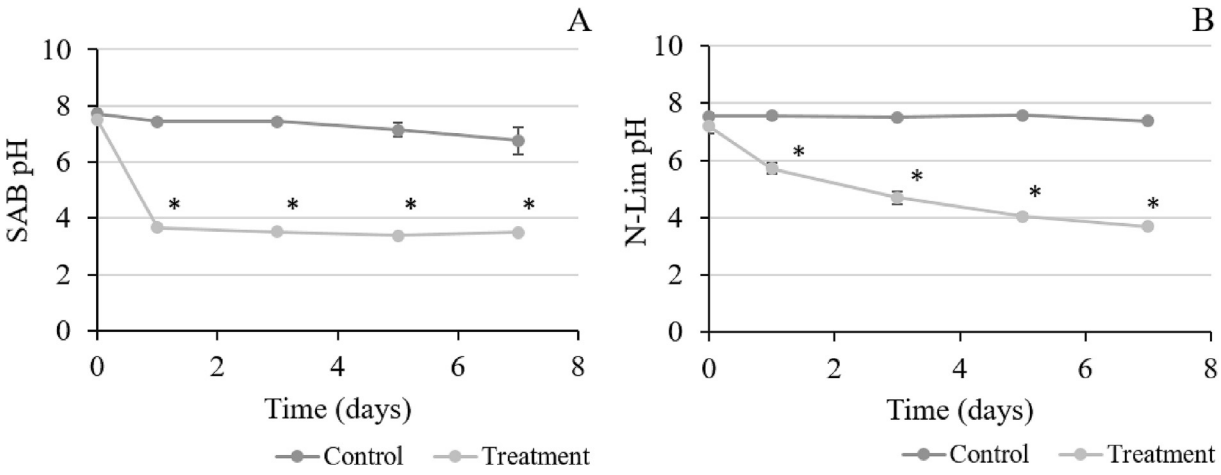

Fig. 2. The pH of A) the SAB medium and B) the N-Lim medium with time in the presence and absence of *M. hiemalis*. Asterisks (*) represent statistical significances compared to the control. Data are presented as mean ± standard deviation ($n = 4$).

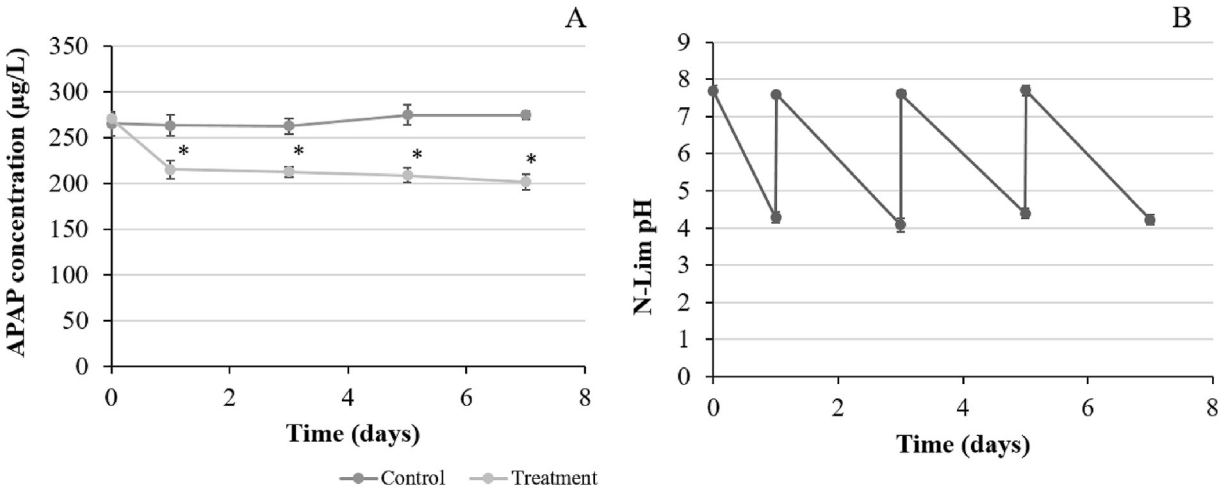

Fig. 3. A) APAP concentration in N-Lim in the presence of *M. hiemalis* with B) daily pH adjustment to 7.5; also showing the actual pH before adjustment per day. Asterisks (*) represent statistical significances compared to the control. Data are presented as mean ± standard deviation ($n = 4$).

between 4 and 7.5, all these enzymes could be active. However, a daily decline in enzyme activity may occur after the optimum pH is exceeded due to partial denaturation because of the dissociation of the ionizable groups of enzymes. This may affect the binding of a substrate to the enzyme (Khalaf et al., 2012). The importance of identifying the enzyme(s) involved thus becomes evident to maintain its optimum pH. Raw wastewater has been reported to have a stable pH varying between 6 and 8, unless the hydrogen sulfide (H_2S) concentration is significant, which reduces it below 6 (TPO, 2013). During tertiary treatment, *Nitrosomonas* spp., which colonize first, thrive at pH 7.5; however, during the nitrification process, the medium becomes acidic due to the biological oxidation of ammonia to nitrite. After that, slow-growing *Nitrobacter* spp. Appear converting nitrite to nitrate again, contributing to a pH change. However, CO_2 typically acts as a buffer (Egli et al., 2003). As mycoremediation is likely to be implemented after tertiary treatment, it was essential to understand the effect of such pH fluctuations, which could influence remediation efficiency. Therefore, daily fluctuations between 4 and 7.5 were considered adequate to evaluate the efficiency in practice.

3.3. pH adjustments with P. chrysosporium

The APAP remediation ability of *P. chrysosporium* (Fig. 4A) was far superior to that of *M. hiemalis* (Fig. 3A) ($p < 0.001$). *P. chrysosporium* was able to remove 97%, and 99% of the APAP (250 µg/L) in its environment after 3 and 7 days, respectively, which was a significant reduction compared to the control ($p < 0.001$) (Fig. 4A). The remediation occurred in N-Lim medium in which the pH decreased to 4 and was not adjusted (Fig. 4B).

However, with pH control, the APAP elimination percentage was reduced to only 23% (Fig. 4A). White-rot fungi metabolize in acidic environments, subsequently decreasing the pH level of the medium (Hung and Trappe, 1983) as seen in Fig. 4B. *P. chrysosporium* produced two lignin peroxidase enzymes in a nitrogen-limited environment (Tuisel et al., 1990), which perform optimally at a pH range of between 4 and 4.5 (Kirk et al., 1978), and could be responsible for the APAP degradation. These enzymes possibly are incapacitated when the pH was adjusted daily, resulting in the 75% reduction in APAP remediation efficiency. Additionally, these enzymes have a temperature optimum between 32 and 34 °C (Rodriguez Couto et al., 2006); thus, the cultivation temperature of 30 °C used may have attributed to the lower efficiency.

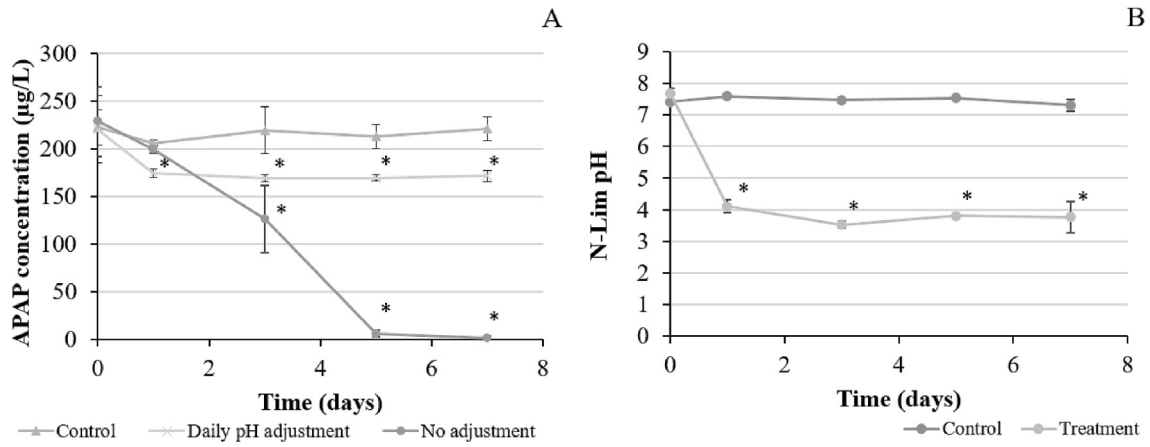

Fig. 4. A) APAP remediation by *P. chrysosporium* in N-Lim medium without daily pH adjustment. B) Medium pH with time. Asterisks (*) represent statistical significances compared to the control. Data are presented as mean ± standard deviation ($n = 4$).

N-Lim media is evidently a suitable medium for *P. chrysosporium*. Eaton (1985) and Jeffries (1990) stated that the depletion of nutrients forces white-rot fungi to degrade lignin to gain access to cellulose components as a carbon source. With *P. chrysosporium*, limitation of nitrogen, carbon, or sulfur (Jeffries et al., 1981) can trigger secondary metabolism and thus enzyme production. Limiting factors such as nitrogen, carbohydrate or sulfur, thus influence the secretion of lignin-degrading enzymes (Crawford, 1981). In domestic wastewater, the total nitrogen concentration varies between 20 and 35 mg/L (Li et al., 2017), which is comparable to the total nitrogen content in N-Lim (50 mg/L).

Interestingly, the hairy region roughness (Krull et al., 2013) of the *P. chrysosporium* pellets become more pronounced, i.e. spiky, with exposure to the APAP both with and without pH control compared to the unexposed controls, which had a smoother appearance.

3.4. Co-cultivation

It was expected that co-cultivation would cause competition for nutrients and thus would result in increased remediation and that the fungi would complement each other's shortcomings. Co-cultivation of *M. hiemalis* and *P. chrysosporium*, however, resulted in a reduced APAP remediation efficiency both with and without pH adjustment (Fig. 5) compared to that achieved with *P. chrysosporium* in single without pH adjustment (Fig. 4A). With co-cultivation, 35% of the APAP was removed with pH adjustment after three days, and 97% after seven days and only 21% after seven days with pH adjustment (Fig. 5).

With pH adjustment, the pH cycled from 4 to 7.5 (Fig. 3B) with daily correction. Therefore, only when the pH was acidic could the *P. chrysosporium* enzymes have been active, and this was a reduced window of time compared to when the pH was unadjusted, which can explain the significantly reduced elimination of the APAP after the first 24 h. When the pH was adjusted to 7.5 daily, *M. hiemalis*'s enzymes were stimulated at the neutral pH; however, as seen in Fig. 3A, these were less efficient than those of *P. chrysosporium*. It should also be noted that the cultivation temperature for co-cultivation was lower than the optimum temperature for *P. chrysosporium* to accommodate *M. hiemalis*. This also could have contributed to a weaker performance by *P. chrysosporium*.

M. hiemalis was previously proven as an excellent candidate for diclofenac remediation (Esterhuizen-Londt et al., 2017) and APAP at concentrations below 20 µg/L (Esterhuizen-Londt et al., 2016a). In the present study, the APAP remediation efficiency of *M. hiemalis* could be improved through pH maintenance and thereby bridge

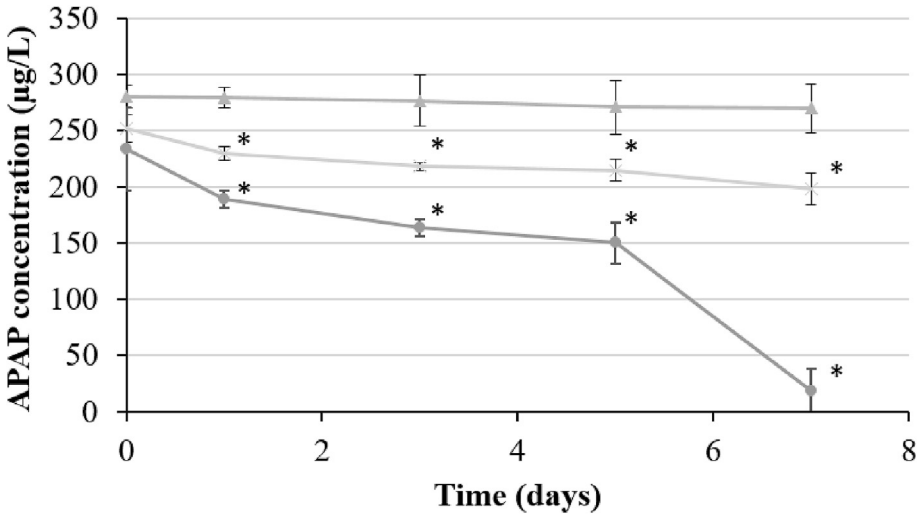

Fig. 5. APAP remediation with co-cultivation of *M. hiemalis* and *P. chrysosporium* in N-Lim medium with and without daily pH adjustment. Asterisks (*) represent statistical significances compared to the control. Data are presented as mean ± standard deviation ($n = 4$).

the 24 h remediation limitation previously seen (Esterhuizen-Londt et al., 2016a). However, the APAP remediation ability of P. *chrysosporium* far exceeded that of *M. hiemalis*, deeming it a better option for the remediation of APAP. Interestingly, co-cultivation did not improve the elimination of APAP. This indicates that an ideal strain for the remediation of a specific contaminant could be paired, and can be compared to a lock and key. However, when requiring remediation for mixtures as is the case in WWTP, the slight reduction in efficiency of co-cultivation is acceptable to achieve a comprehensive remediation technique. Therefore, by accepting the reduction in efficiency for APAP with co-cultivation, the technique becomes applicable not only for the remediation of one pharmaceutical. The technique could be extended to encompass strains that co-remediate complex mixtures, not only limited to pharmaceutical pollution, thereby establishing this research as a foundation for future developments.

4. Conclusion

Mycoremediation has the potential to serve as a sustainable, effective, and environmentally friendly remediation tool against a vast range of environmental pollutants. However, screening of strains against pollutants and culture parameter optimization, as well as finding the most practical combination of fungi to treat an environmental pollution issue are needed to improve mycoremediation as a practical tool for water purification. In the present study, we show that nitrogen limitation and pH control indeed improve the remediation efficiency of *M. hiemalis*; however, *P. chrysosporium was* far superior. Interestingly, co-cultivation decreased the remediation efficiency; yet this small loss could be accepted in the broader scope of developing a comprehensive technology applicable to a wide range of conditions. With the knowledge regarding APAP remediation gained during the present study, in conjunction with known data regarding diclofenac remediation by *M. hiemalis* and remediation of carbamazepine, tetracycline, and oxytetracycline by *P. chrysosporium*, co-cultivation of the two species can be considered an effective paring for testing and implementation in WWTPs. However, the practicality of mycoremediation in the environment and biotechnological applications needs to be investigated at a larger scale.

Credit author statement

Maranda Esterhuizen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, writing original draft, review and editing; Lin Wang: Investigation, Methodology, Formal analysis, Writing - review & editing; Shirin Behnam Sani: Investigation, Methodology, Formal analysis, Writing - review & editing; Young Jun Kim: Investigation, Methodology, writing-review and editing; Stephan Pflugmacher: Conceptualization, Funding acquisition, Supervision, writing-review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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